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Inducible T Cell Antigen 4–1BB[1]

Analysis of Expression and Function

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Abstract. 4–1BB is an inducible receptor-like protein expressed in both cytolytic and Th cells. Optimal induction of 4–1BB mRNA in T cells required both PMA and ionomycin stimulation, indicating that protein kinase C activation and increases in intracellular Ca^{2+} were required for its expression. 4–1BB was categorized as an early activation gene since the protein synthesis inhibitor, cycloheximide, blocked the induction of 4–1BB mRNA. A rat mAb, 53A2, was generated against recombinant soluble 4–1BB and was used to characterize this molecule. 4–1BB is a 30-kDa glycoprotein and appears to exist as both a monomer and a 55-kDa dimer on the cell surface of a T cell clone. The 4–1BB protein may be post-translationally modified since its predicted backbone is 25 kDa. FACS analysis indicated that 4–1BB was inducible and expressed on the cell surface of activated splenic T cells and thymocytes. Cross-linking of 4–1BB on anti-CD3-stimulated T cells with 53A2 resulted in a dramatic enhancement of T cell proliferation. This suggests that 4–1BB may function as an accessory signaling molecule during T cell activation. *Journal of Immunology,* 1993, 150: 771.

Our laboratory previously reported a series of T cell subset-specific cDNA isolated from cloned murine T cells by employing a modified differential screening procedure (1). The nucleotide sequence and expression properties of some of the cDNA species have been reported (2). One of these genes not previously characterized, 4–1BB, was studied further. The 4–1BB cDNA encodes a peptide of 256 amino acids containing a putative leader sequence, a potential membrane anchor segment, and other features of known receptor proteins (2). The transcript of 4–1BB was previously found to be inducible by Con A in mouse splenocytes, T cell clones, and hybridomas. The expression of 4–1BB transcripts was inhibited by cyclosporin A. In cloned T cells, 4–1BB mRNA was inducible by Ag receptor stimulation, but not by IL-2 stimulation (3). Therefore, the expression patterns of 4–1BB resemble those of lymphokine mRNA, whereas the sequence appears consistent with that of receptor proteins.

Recently, a number of cysteine-rich receptor proteins have been described and named as the NGFR[3] superfamily (4). At present, members of the NGFR superfamily include NGFR (5); the B cell Ag CD40 (6); the MRC OX-40 Ag (7), which is a marker of activated T cells of the CD4 phenotype; two receptors for TNF called TNFR-I and TNFR-II, which are found on a variety of cell types (8, 9); SFV-T2 (10); an open reading frame in the Shope fibroma virus, which later was identified as a virally encoded, soluble form of TNFR-I; a T cell surface Ag, CD27, which may be involved in T cell activation (11); Fas, a cell surface Ag that can mediate apoptosis (12); Sal F19R, an

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[3] Abbreviations used in this paper: NGFR, nerve growth factor receptor; PMA, phorbol 12-myristate 13-acetate; Sf-21, *Spadoptera frugiperda*-21; AcNPV, *Autographa californica* nuclear polyhedrosis virus; 4–1BBP, 4–1BB protein; rs-4–1BBP, recombinant soluble 4–1BB protrein.

open reading frame in the Shope sarcoma virus (13); and 4–1BB, which is the most distantly related member. The newest member is CD30, a Hodgkin's lymphoma Ag that may play a role in the regulation of cellular growth and transformation (14). Members of the family are characterized by the presence of three to six patterns of a cysteine-rich motif that consist of about 40 amino acids in the extracellular part of the molecule. These molecules contain a hinge-like region immediately adjacent to the transmembrane domain. This region is characterized by a lack of cysteine residues and a high proportion of serine, threonine, and proline, which are likely to be glycosylated with O-linked sugars.

4–1BB contains other interesting features in its cytoplasmic domain. These include: 1) two runs of acidic amino acids; 2) a potential $p56^{lck}$ binding site; 3) five consecutive glycines at the carboxyl terminus; and 4) four potential phosphorylation sites—one tyrosine, two threonine, and one serine. It is especially interesting that 4–1BB contains a potential $p56^{lck}$ binding site, -C-R-C-P-. The consensus sequence for the $p56^{lck}$ binding site is -C-X-C-P- in the CD4 and CD8 molecules (15).

To address the biologic function of 4–1BB, we prepared a mAb, 53A2, which recognizes the 4–1BB protein. This reagent was used to characterize biochemical and expression properties of 4–1BB as well as assess the role of 4–1BB signaling in T cells.

Materials and Methods

Cells

CTLL-R8, a mouse cytolytic T cell line, was grown in DMEM (Gibco Laboratories, Grand Island, NY) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 4 U/ml of rIL-2 (Boehringer-Mannheim, Indianapolis, IN), and 10% FBS. F1 is a $CD4^+$, $I\text{-}A^d$-reactive T cell clone that was generously provided by Dr. Scott Bryson, University of Kentucky. This clone was isolated from DBA/2 mice that had syngeneic graft vs host disease. This clone was maintained in RPMI 1640 (Gibco Laboratories) containing 10% FBS, 50 μM 2-ME, and antibiotics (complete medium). Rat Con A supernatant was added at 30%. Sf-21, an insect cell line, was grown in synthetic serum-free Ex-cell 400 medium (JRH Biosciences) containing antibiotics at 27°C (16).

Preparation of splenic T cells

The resting murine splenic T cells were enriched by nylon wool and Percoll gradient centrifugation (17, 18). Briefly, the spleen cells of female BALB/c mice (Harlan, Indianapolis, IN) were adjusted to 1.5×10^8/ml in RPMI 1640 containing 2% FBS. A 10 ml nylon wool column was prewashed with PBS, pH 7.4, followed by RPMI 1640 containing 2% FBS, and incubated at 37°C for 20 min before use. The spleen cells were loaded onto the column and incubated at 37°C for 30 min. The column was washed with the above medium. The eluted cells subsequently were fractionated by centrifugation at $2000 \times g$ for 30 min at 4°C on a four-step gradient consisting of 80% (1.08 g/ml), 70% (1.07 g/ml), 60% (1.06 g/ml), and 50% (1.05 g/ml) Percoll solutions (Pharmacia, Piscataway, NJ). The resting T cell fraction was recovered from the interface between the 60% and 70% Percoll layers. The enriched T cells were examined with an EPICS Profile Analyzer (Coulter Corp.); ~91% were Thy 1.2^+, ~52% were $L3T4^+$, and ~24% were Lyt 2^+. These cells exhibited a uniform low degree of forward angle light scatter.

Thymocyte preparation

Thymuses from 6- to 8-wk old BALB/c mice were aseptically removed and teased into a cell suspension. Thymocytes were washed twice in RPMI 1640 containing 2% FBS.

Production of recombinant 4–1BB protein in Sf-21 cells

To construct a plasmid that expresses the extracellular portion of 4–1BB, the putative extracellular domain of 4–1BB cDNA (2) was amplified by the polymerase chain reaction (19). An *Xho*I site was created at the 5′ end of the forward primer, while a stop codon (TAA) and an *Eco*RI site were created in the reverse primer. The polymerase chain reaction product was digested with *Xho*I and *Eco*RI, and the ~0.6-kb fragment was purified. The *Xho*I-*Eco*RI fragment (4–1BBS) was inserted into the PEV-55 vector (16), generating PEV-55–4–1BBS. The sequence of the forward primer was 5′-ACCTCGAGGTCCTGTGCATGTGACA-3′, and that of the reverse primer was 5′-ATGAATTCTTACTGCAAGGAGTGCCC-3′.

To express the entire 4–1BB, a 1.2-kb cDNA fragment (4–1BBL) that contains all the coding sequence of 4–1BB was inserted into the *Eco*RI site of the PVL 1392 vector (a kind gift from Dr. Max Summers), generating PVL 1392–4–1BBL.

4–1BBS, as well as 4–1BBL, were transferred from these plasmids to the AcNPV genome by cotransfection into Sf-21 cells as described (16). Ten occlusion-negative viruses for each construct were plaque-purified. AcNPV-4–1BBS and AcNPV-4–1BBL recombinant viral stocks were grown in Sf-21 cells in serum-free Ex-cell 400 medium.

Rabbit polyclonal antiserum against 4–1BB oligopeptides

Five oligopeptides representing different regions of the deduced 4–1BBP sequence were synthesized (Applied Biosystems, Foster City, CA). Two sequences, named

4–1BB-0 and 4–1BB-11, stimulated the production of antibodies. The amino acid sequence of the oligopeptide 4–1BB-0 was a 12-mer from amino acids 105 to 115 of the deduced 4–1BBP (2). Oligopeptide 4–1BB-11 was a 25-mer from amino acids 133 to 157 of the deduced 4–1BBP (2). A tyrosine residue at the C-terminus of the oligopeptide 4–1BB-0 was added for labeling with [^{125}I] if needed. The peptides were conjugated to keyhole limpet hemocyanin using a heterobifunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (20).

Rabbits were immunized with peptide-keyhole limpet hemocyanin (100 μg/dose) emulsified in CFA. The rabbits received one intracutaneous injection in each of four foot pads and one i.m. injection 2 wk apart. After 2 wk, the rabbits received three consecutive i.v. injections (50 μg/dose) without adjuvant. The serum was obtained 5 days after the final injection, and the titer was measured by ELISA.

Purification of the rs-4–1BBP

The serum-free culture supernatants of Sf-21 cells infected by AcNPV-4–1BBS were concentrated by ammonium sulfate precipitation (60% saturation). The precipitate was dissolved in a buffer containing 40 mM Tris-HCl (pH 7.8), 50 mM NaCl, and 0.02% NaN_3. This resulting solution was dialyzed overnight at 4°C against the same buffer. The sample was subsequently fractionated in a Sephacryl S-300 column equilibrated in the above buffer. The fractions containing 4–1BBS were identified by Western blotting with anti-4–1BB-0 antibodies. rs-4–1BBP-containing fractions were pooled and concentrated by vacuum dialysis. The sample was again fractionated over a Q-Sepharose column by elution with a linear gradient of 0 to 1.0 M NaCl. rs-4–1BBP-containing fractions were pooled and dialyzed against 5 mM sodium phosphate buffer, pH 7.2. The samples were loaded on hydroxylapatite column and eluted with a linear gradient of 5 to 100 mM sodium phosphate buffer, pH 7.2. Finally, the rs-4–1BBP-containing fractions were pooled, equilibrated with 50 mM sodium phosphate buffer (pH 7.2), and loaded on a S-Sepharose column. A linear gradient from 0 to 0.5 M NaCl was used to elute the absorbed proteins. The rs-4–1BBP-containing fractions were pooled and concentrated by vacuum dialysis.

Production of anti-4–1BB mAb

Eight week-old Sprague-Dawley rats were immunized with 50 μg of rs-4–1BBP emulsified in Titermax (cytRX). Two s.c. injections separated by a 2-wk interval were administered initially. A third i.m. injection was given 2 wk after the last immunization. Three days after the final injection, the rat spleen was removed. Spleen cells were fused with SP2/0 mouse myeloma cells and cultured according to the standard method (21). ELISA was used to screen for the rs-4–1BBP-reacting clones. Seventeen clones were isolated and subcloned. One clone 53A2 (IgG1) was characterized and used in the present studies. The mAb was purified from culture supernatant by affinity chromatography on protein G-Sepharose (Schleicher-Schuell).

Immunoblot analysis

Cells were washed in PBS and lysed by adding a buffer of 50 mM Tris HCl, pH 8.0, 1% Nonidet P-40, 2 mM EDTA on ice for 15 min. This buffer contained the protease inhibitors aprotinin and leupeptin at 100 μg/ml each. The cell lysate was harvested and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant containing approximately 1 mg/ml of protein was denatured by boiling for 2 min in a sample buffer consisting of 62.5 mM Tris HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-ME, and 0.001% bromphenol blue. The proteins were resolved by electrophoresis on a 12% SDS-polyacrylamide gel and transferred electrophoretically onto an Immobilon membrane (Millipore, Bedford, MA). The membranes were blocked to prevent nonspecific antibody binding by incubating in 5% nonfat dry milk in 50 mM Tris HCl, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20 for 1 h at room temperature. The membranes were then treated with primary antibodies at room temperature for 1 h with gentle agitation. After four washes with 50 mM Tris HCl, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20, the membranes were incubated with a secondary antibody against rabbit or rat IgG(H+L)-alkaline phosphatase conjugate (Zymed, Inc., South San Francisco, CA) at 1:1000 dilution. The reactive bands were visualized by incubating the membrane with chromogenic substrates, *p*-nitroblue-tetrazolium chloride, and 5-bromo-4-chromo-3-indolyl-phosphate (Bio-Rad, Richmond, CA) in 0.1 M Tris, pH 9.5, 0.1 M NaCl, and 5 mM $MgCl_2$.

Immunoprecipitation of cell surface 4–1BB

F1 cells were labeled with [^{35}S]cysteine (Amersham, Arlington, MA) at a concentration of 0.1 mCi/ml for 14 h in a cysteine-free RPMI 1640 and 5% dialyzed FBS. Normal and anti-CD3-stimulated F1 cells were washed twice with PBS and resuspended in PBS containing 0.2% BSA and 0.1% NaN_3. To detect the cell surface 4–1BB Ag, cells were first incubated with 10 μg of 53A2 or unrelated rat IgG1 (Zymed) at 4°C for 1 h. The cells were then recovered, washed, and lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% digitonin, 1 mM sodium vanadate, 5 μg/ml aprotinin, and 1 μg/ml leupeptin) on ice for 15 min. The 4–1BB and 53A2 complexes were precipitated by rec-protein G Sepharose-4B (Zymed). The immunoprecipitates were run on a 10% SDS-polyacrylamide gel

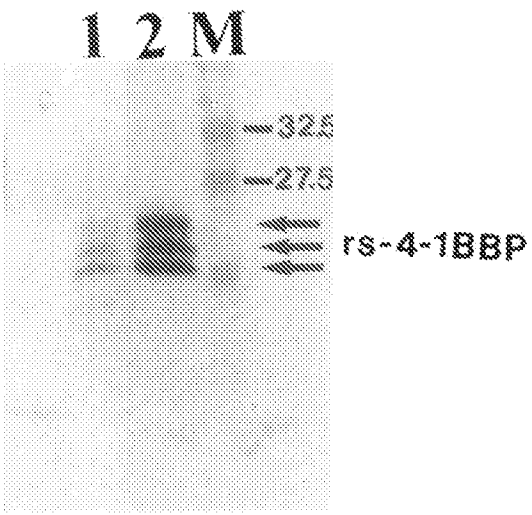

FIGURE 1. Purified rs-4–1BBP. Extracellular domain of 4–1BB was expressed in insect cells (sf 21) using recombinant baculoviruses containing the 4–1BB. rs-4–1BBP has been purified to homogeneity by column chromatography. rs-4–1BBP consists of three bands of 18, 20, and 23 kDa (*arrows*). *Lane 1*, Coomassie staining; *lane 2*, anti-4–1BB-0 antibody staining; *lane 3*, molecular size marker.

and exposed to x-ray film after soaking the gel in EN^{3}HANCE (New England Nuclear).

Flow cytometry

Cells (0.5×10^6/sample) were incubated in RPMI 1640, 1% BSA, and 0.1% sodium azide with purified 53A2 on ice for 30 min. Cells were washed three times in RPMI 1640 containing 5% FBS. The cells were then incubated on ice for 30 min with FITC-conjugated goat anti-rat Ig. Some samples received FITC-conjugated goat anti-rat Ig only.The cells were washed three times with PBS (pH 7.4) containing 1% BSA. Flow cytometry was performed using an EPICS 753 (Coulter) FACS.

Northern blot analysis

Thymocytes or splenic T cells were stimulated with 10 μg/ml immobilized anti-CD3 mAb (clone 145–2C11), 1 μM ionomycin (Calbiochem, La Jolla, CA), 10 ng/ml PMA (Sigma, St. Louis, MO), or ionomycin plus PMA. Individual wells of 96-well flat bottomed culture plates or T25 flasks (Costar, Cambridge, MA) were coated with 10 μg/ml anti-CD3 in PBS, pH 7.0, for 3 h at 37°C and then washed three times with PBS. For RNA extraction, 2 to 3×10^7 cells at 5×10^6/ml were stimulated in complete medium and harvested at the indicated time points. Total RNA was extracted by the guanidinium-phenol extraction procedure (22). The RNA was fractionated on a 1.4% formaldehyde-denaturing agarose gel, transferred to a Gene-Screen Plus membrane, and hybridized with ^{32}P-labeled probes.

Results

Specificity of anti-oligopeptide antisera to 4–1BB and expression of 4–1BBP

The only information previously available regarding 4–1BB was the nucleotide sequence of the cDNA and the predicted amino acid sequence. To study the 4–1BBP, we raised polyclonal antibodies against oligopeptides representing five different portions of the predicted 4–1BBP. To

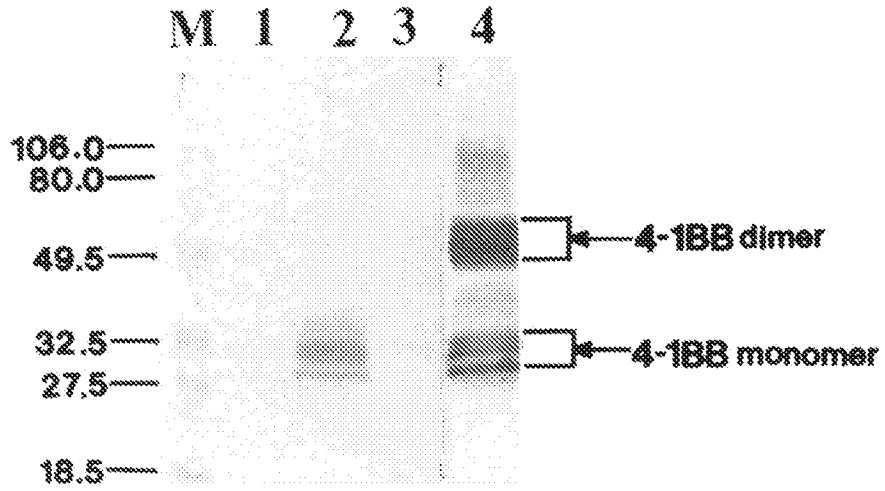

FIGURE 2. Recombinant 4–1BB protein. Full length 4–1BB cDNA (4–1BBL) was expressed in insect cells (sf 21) using recombinant baculoviruses containing 4–1BBL. The cell lysates were fractionated on a 10% SDS polyacrylamide gel, transferred to Immobilon-p, and stained with 53A2, an anti-4–1BB mAb. The monomer of 4–1BB in reducing conditions consists of three major species whose sizes are 29, 30 and 31 kDa (*lane 2*). Nonreducing conditions (*lane 4*) produced dimerized 4–1BB, whose sizes are 49 to 60 kDa. Some of 4–1BB molecules are monomerized in *lane 4*, showing both monomers and dimers. *Lane 1*, uninfected Sf 21 cell lysate; *lane 2*, 4–1BBL-infected Sf 21 cell lysate; *lane 3*, MIP-1β-infected Sf 21 cell lysate (unrelated to 4–1BBL); *lane 4*, 4–1BBL-infected Sf 21 cell lysate.

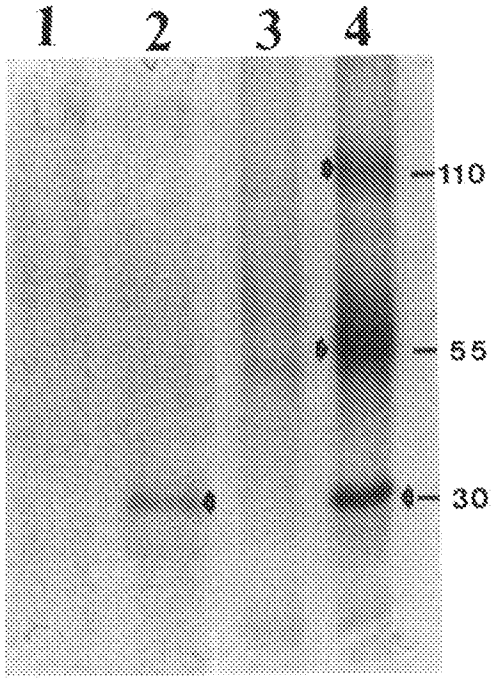

FIGURE 3. Immunoprecipitation of cell surface 4–1BB protein synthesized by T lymphocytes. T cell (F1 clone) surface 4–1BB protein was immunoprecipitated with anti-4–1BB mAb 53A2 (*lanes 2* and *4*) or with rat IgG1 control mAb (*lanes 1* and *3*). *Lanes 1* and *2* were run under reducing conditions and *lanes 3* and *4* were run under nonreducing conditions. Monomeric form of 4–1BB protein is indicated by an *arrowhead with number 1*. The dimeric and tetrameric forms are indicated by the *arrowheads with numbers 2* and *3*, respectively.

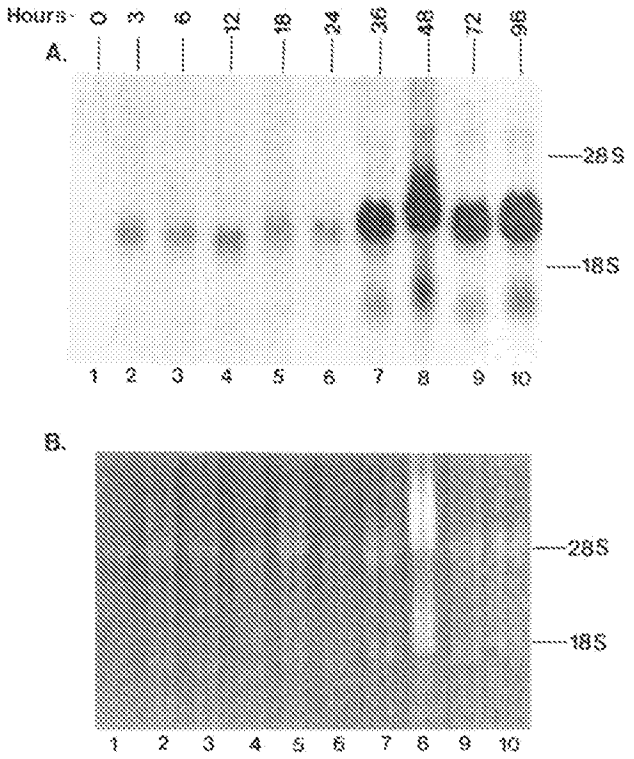

FIGURE 4. 4–1BB mRNA expression is induced in murine splenic T cells by anti-CD3 activation. Splenic T cells were isolated by a nylon wool column and a Percoll gradient (cells were isolated at the 60 to 70% interface) and stimulated over time with 10 μg/ml of immobilized anti-CD3 (145–2C11) in the presence of accessory cells. Cultures were harvested at indicated time points and monitored for 4–1BB expression by Northern analysis. Each *lane* contains 20 μg of total RNA. Northern blot was hybridized with a 4–1BB cDNA probe (3A) and the gel was stained with ethidium bromide before transfer as a control for equal loading of each lane (3B).

aid in proving that the putative antisera contained antibodies that uniquely recognized the 4–1BBP, we constructed an expression plasmid containing the putative extracellular portion of 4–1BB cDNA. The truncated cDNA was expressed in a baculoviral expression system. We then tested whether any of our antibodies recognized rs-4–1BBP in the Sf-21 cell culture medium. Two antisera among five tested recognized a protein found only in the supernatant of 4–1BBS-infected cells. The anti-oligopeptide antiserum, anti-4–1BB-0, stained the recombinant band at the highest titer (1:1600). rs-4–1BBP was purified from Sf-21 cell culture medium as described in *Materials and Methods*. As shown in Figure 1 (*arrows*), we detected anti-rs-4–1BBP-reacting bands of approximately 18, 20, and 23 kDa (Fig. 1: *Lane 1*, Coomassie blue staining; *Lane 2*, anti-4–1BB-0 staining). Partial amino acid sequences of each of the three bands were determined using an automatic peptide sequencer. The sequences of the three bands were identical and were Val-Gln-Asn-Ser-X-Asp. The amino acid sequence at positions 1, 2, 3, 4, and 6 was identical to that of the mature 4–1BBP predicted from the cDNA sequence. Amino acid at position 5 that was supposed to be Cys was not confirmed. Production of three different sizes of rs-4–1BBP is most likely due to the differences in glycosylation. These results suggest that the deduced amino acid sequence and assignment of the signal sequence were correct. When the potential transmembrane domain was removed from the complete 4–1BB molecule, the protein was secreted, which is consistent with the full length 4–1BBP being associated with the cellular membrane as predicted by the primary structure.

Biochemical properties of 4–1BB

Full length 4–1BB cDNA (4–1BBL) was expressed in Sf-21 cells using recombinant baculoviruses containing 4–1BBL. The cell lysates were fractionated on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P. The blot was incubated with 53A2, an anti-4–1BB mAb, and stained with goat anti-rat IgG-conjugated alkaline phosphatase. Under reducing conditions, the 4–1BB monomer consisted of three major species corresponding to 29, 30, and 31 kDa (Fig. 2, *Lane 2*). Under nonreducing

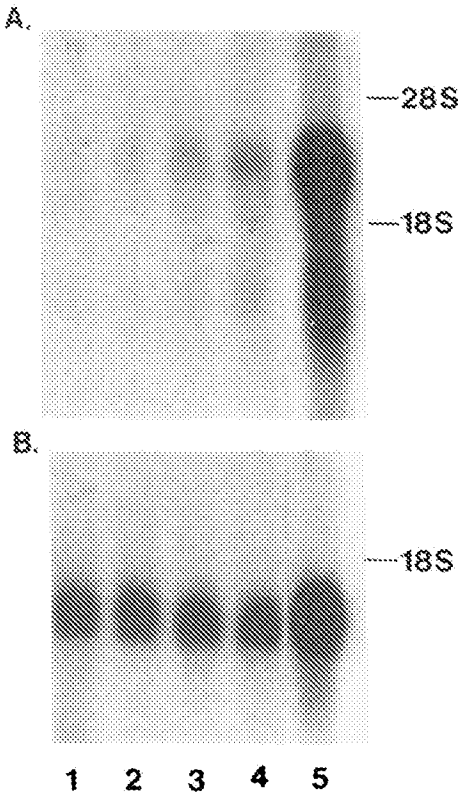

FIGURE 5. Optimal induction of 4–1BB mRNA requires both protein kinase C activation and increases in intracellular Ca^{++}. Thymocyte cultures were stimulated with medium alone (*lane 1*), 1 μM ionomycin (I) (*lane 2*), 10 ng/ml TPA (T) (*lane 3*), T + I (*lane 4*) for 6 h, and monitored for 4–1BB mRNA expression by Northern analysis. RNA was also isolated from CTLL-R8 cells and monitored for 4–1BB mRNA by Northern analysis (*lane 5*). Each *lane* contains 10 μg of total RNA. Blot was hybridized sequentially with a 4–1BB cDNA probe (4A) and with a CHOB probe (4B) as a control for equal loading in each *lane*.

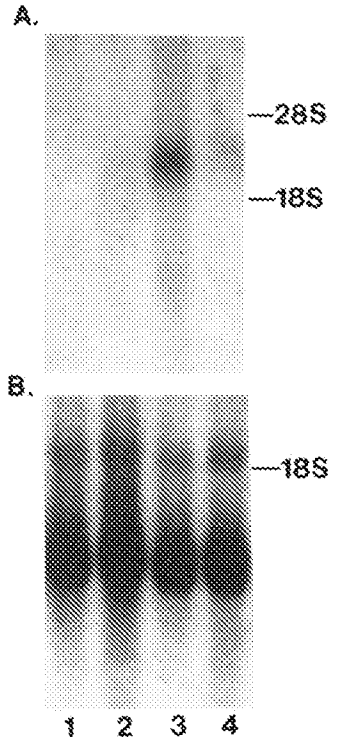

FIGURE 6. Transacting factors necessary for the induction of 4–1BB mRNA expression do not pre-exist in the resting T cell. Cycloheximide (CHX), a protein synthesis inhibitor, blocks the induction of 4–1BB mRNA in TPA and ionomycin-activated thymocytes. Thymocytes cultures were stimulated with medium alone (*lane 1*), 20 μg/ml CHX (*lane 2*), T + I (*lane 3*), or CHX + T + I (*lane 4*) for 6 h and monitored for 4–1BB mRNA expression by Northern blot analysis. Each *lane* contains 10 μg of total RNA. Blot was hybridized sequentially with a 4–1BB-cDNA probe (4A) and with a CHOB cDNA probe (4B) as a control for equal loading in each *lane*.

conditions, 4–1BB existed as a monomer as well as a dimer of 49 to 60 kDa. The heterogeneity in size may be due to different amounts of glycosylation as seen in rs-4–1BBP (Fig. 1).

4–1BBP expressed on T lymphocytes was immunoprecipitated with the 53A2 mAb. A $CD4^+$ T cell clone F1 was labeled with [^{35}S]cysteine. The 53A2 mAb (Fig. 3, *Lanes 2* and *4*) or rat IgG1 control (Fig. 3, *Lanes 1* and *3*) antibodies was first incubated with F1 cells to bind to surface 4–1BBP. The cells were subsequently washed to remove unbound antibody, lysed, and immunoprecipitated with protein G-conjugated Sepharose beads. Under reducing conditions, 53A2 immunoprecipitated a 30-kDa protein (Fig. 3, *arrow 1*), and under nonreducing conditions, 53A2 immunoprecipitated 30-kDa (Fig. 3, *arrow 1*), 55-kDa (Fig. 3, *arrow 2*), and 110-kDa (Fig. 3, *arrow 3*) proteins. These three bands may represent monomer, dimer, and tetramer species, respectively. Whether all three of these forms of 4–1BBP are in a dynamic equilibrium on the cell surface is not known. The most abundant form appears to be the dimeric 4–1BB.

Expression of 4–1BBP in splenic T cells and thymocytes

Splenic T cells were isolated by a nylon wool column and a subsequent Percoll gradient. The T cells were stimulated with 10 μg/ml of immobilized anti-CD3 mAb. T cells were harvested at various time points and tested for 4–1BB mRNA expression by Northern blot analysis. As shown in Figure 4, 4–1BB mRNA was detected by 3 h of stimulation. Northern analysis also indicated that 4–1BB mRNA was not expressed in LPS-activated B cell blasts or in A20 cells, a B cell lymphoma (data not shown).

Thymocytes were stimulated with ionomycin, PMA, or ionomycin plus PMA and tested for 4–1BB mRNA expression. As shown in Figure 5, 4–1BB mRNA was inducible by ionomycin or PMA. Both ionomycin and PMA, however, were required for optimal expression of 4–1BB mRNA, indicating that 4–1BB mRNA expression requires

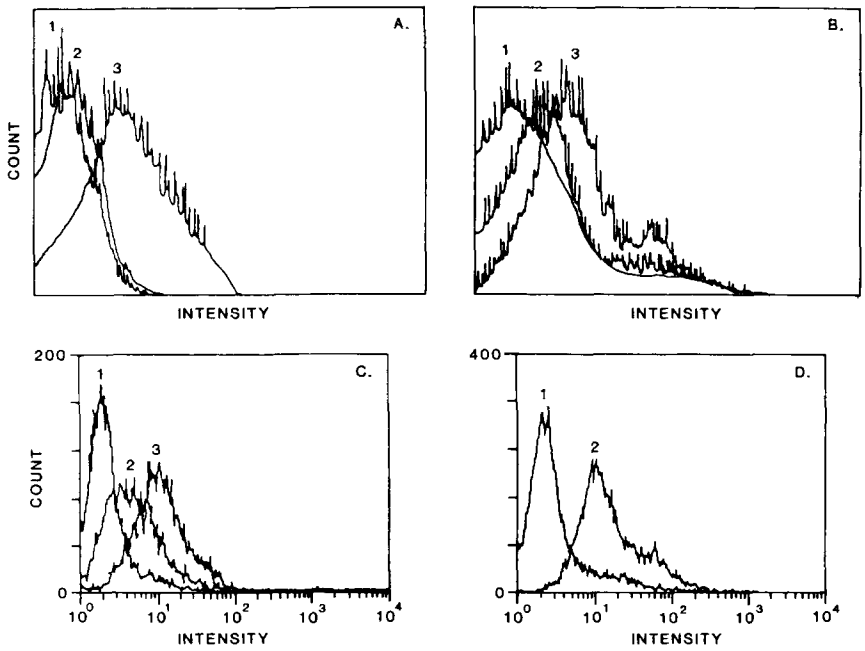

FIGURE 7. 4–1BB is expressed on the cell surface of activated thymocytes, splenic T cells, $CD4^+$, and $CD8^+$ T cells. *A*, Thymocytes were stained with FITC-conjugated goat anti-rat IgG (anti-IgG-FITC) alone (*peak 1*) or with anti-4–1BB mAb plus anti-IgG-FITC on control thymocytes (*peak 2*), or thymocytes stimulated with T + I for 12 h (*peak 3*). *B*, Splenic T cells were stained with anti-IgG-FITC alone (*peak 1*) or with anti-4–1BB mAb plus anti-IgG-FITC on resting T cells (*peak 2*) or T cells stimulated with immobilized anti-CD3 for 12 h (*peak 3*). *C*, A $CD4^+$ T cell clone, F1, was stained with anti-IgG-FITC alone (*peak 1*) or with anti-4–1BB mAb plus anti-IgG-FITC on control F1 cells (*peak 2*), or F1 cells stimulated with immobilized anti-CD3 for 24 h (*peak 3*). *D*, A $CD8^+$ T cell line, CTLL-R8, was stained with anti-IgG-FITC alone (*peak 1*) or with anti-4–1BB mAb plus anti-IgG-FITC (*peak 2*).

protein kinase C activation and increases in intracellular Ca^{2+}. The induction of 4–1BB mRNA was blocked by cycloheximide treatment, suggesting that 4–1BB mRNA expression requires new protein synthesis (Fig. 6). Therefore, 4–1BB is classified as a member of the early gene family (23).

FACS analysis indicated that 4–1BBP was expressed on the surface of activated thymocytes, splenic T cells, $CD4^+$, and $CD8^+$ T cells. As shown in Figure 7, 4–1BBP was inducible when the thymocytes were treated with ionomycin plus PMA (Fig. 7*A*, *peak 3*), and when the splenic T cells were treated with anti-CD3 mAb (Fig. 7*B*, *peak 3*). The 4–1BBP was also induced in the $CD4^+$ T cell clone, F1, by anti-CD3 mAb (Fig. 7*C*, *peak 3*) and was expressed constitutively in CTLL-R8 cells (Fig. 7*D*, *peak 2*).

Effect of the anti-4–1BB mAb, 53A2, on anti-CD3-stimulated T cell proliferation

To determine whether biochemical signals delivered through 4–1BB may contribute to T cell activation, the anti-4–1BB mAb, 53A2, was used to mimic ligand binding to cell surface 4–1BB. Purified resting splenic T cells were stimulated with 10 μg/ml immobilized anti-CD3 in the absence or presence of 53A2. In Figure 8, on days 3 to 5 of activation, we observed a four- to sixfold enhancement of [^{3}H]thymidine incorporation in T cells stimulated with anti-CD3 in the presence of 53A2. Enhancement of proliferation was not present when T cells were stimulated with anti-CD3 in the presence of a rat isotype-matched control antibody. In data not shown, the 4–1BB anti-peptide antiserum, 4–1BB-O, had no effect on anti-CD3-mediated T cell proliferation. In other experiments, the actual degree of enhancement ranged from an approximately 2- to 10-fold increase in [^{3}H]thymidine incorporation in cultures stimulated with anti-CD3 and 53A2 compared to anti-CD3 alone (Table I). Differences in actual enhancement of [^{3}H]thymidine incorporation could be due to variability in the number or metabolic status of accessory cells in the cultures. For example, the highest -fold increase in T cells stimulated with anti-CD3 in the presence of 53A2 generally occurred when the proliferative effects of anti-CD3 were minimal (in Table I, compare day 3 of experiments I and II). It will be necessary to obtain T cell populations of higher purity to address this issue. These data, however, conclusively show that 4–1BB-mediated signals can contribute to T cell proliferation.

Discussion

These experiments represent an initial characterization of 4–1BB and propose that 4–1BB may function as a cell

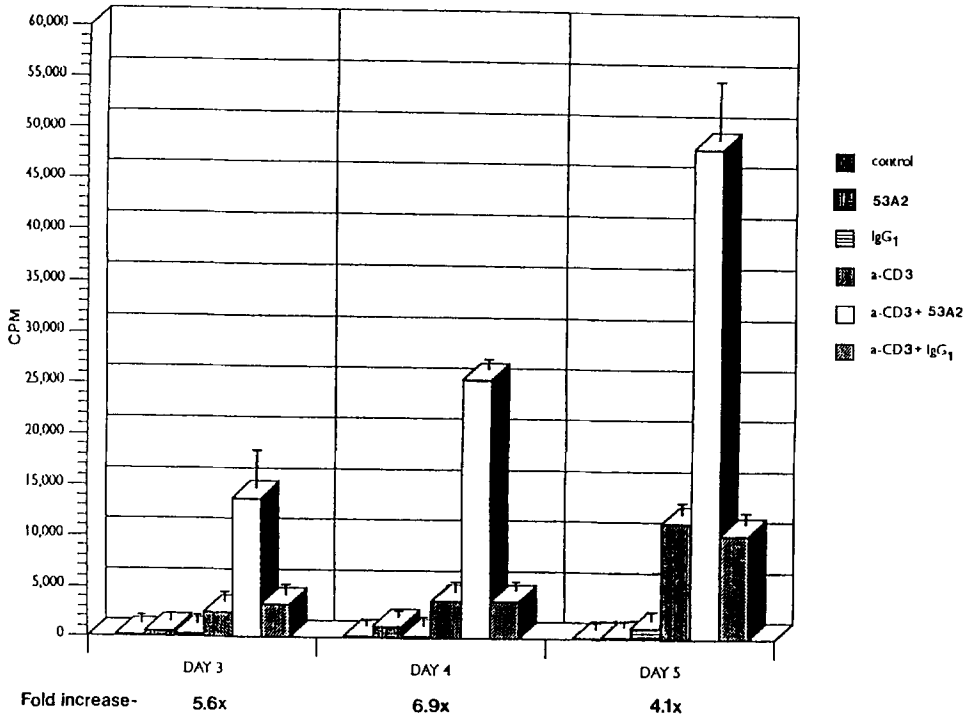

FIGURE 8. Anti-4–1BB mAb, 53A2, enhances the proliferation of anti-CD3-activated splenic T cells. Purified splenic T cells (5×10^4 cells/well) were stimulated in triplicate with 10 μg/ml immobilized anti-CD3 alone or in the presence of 10 μg/ml 53A2 or 10 μg/ml rat IgG1 as a control. Cultures were incubated for the indicated times and pulsed for 12 to 14 h with 1 μCi [^{3}H]thymidine. The cultures were harvested and [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

Table I
Enhancement of anti-CD3-stimulated T cell proliferation by the anti-4–1BB mAb 53A2[a]

	[^{3}H]TdR incorporation					
	Experiment I			Experiment II		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
Control	320 ± 57	715 ± 74	790 ± 117	336 ± 185	284 ± 35	120 ± 26
53A2 (10 μg/ml)	1103 ± 60	2268 ± 232	3323 ± 717	387 ± 69	326 ± 36	166 ± 71
IgGl (10 μg/ml)	710 ± 95	1501 ± 165	1553 ± 196	381 ± 224	302 ± 87	229 ± 85
αCD3 (10 μg/ml)	3164 ± 714	11,217 ± 735	17,380 ± 420	16,999 ± 971	15,823 ± 209	6,877 ± 1176
αCD3 + 53A2 (10 μg/ml)	44,155 ± 665	35,796 ± 370	60,443 ± 803	30,570 ± 1250	29,875 ± 343	10,668 ± 1165
αCD3 + IgGl (10 μg/ml)	7,945 ± 1217	10,404 ± 1999	17,843 ± 2490	17,638 ± 3274	15,365 ± 223	6,238 ± 110
Fold increase αCD3 vs αCD3 + 53A2	9.6×	3.0×	3.5×	1.8×	1.9×	1.6×

[a] The anti-4–1BB mAb 53A2 enhances proliferation of anti-CD3-activated splenic T cells. Purified splenic T cells (5×10^4 cells/well) were stimulated in triplicate with 10 μg/ml immobilized anti-CD3 alone or in the presence of 10 μg/ml 53A2 or 1 μg/ml rat IgG1 as a control. Cultures were incubated for the indicated times and pulsed for 12 to 14 h with 1 μCi [^{3}H]TdR. The cultures were harvested and [^{3}H]TdR incorporation was measured by liquid scintillation counting.

surface receptor capable of transmitting biochemical signals during T cell activation.

The major species of 4–1BB on the cell surface appears to be a 55-kDa dimer. 4–1BB also appears to exist as a 30-kDa monomer and possibly as a 110-kDa tetramer. Since these 4–1BB species were immunoprecipitated from a homogenous population of cells (T cell clone F1), all forms potentially coexist on each cell. We are currently determining whether these forms also exist on primary cultures of T cells activated with anti-CD3. A comparison of peptide digests from the 4–1BB monomer and dimer will be needed to determine whether 4–1BB exists as a homodimer on the cell surface. A variety of cell surface receptors such as the insulin receptor (24), the B cell surface Ig receptor (25), the T cell Ag receptor (26), the CD28 costimulatory receptor (27), and the CD27 T cell Ag (28) are composed of disulfide-bonded subunits. Receptor dimerization may be required for ligand binding and subsequent biochemical signaling through these particular receptors.

4–1BB is not expressed on resting T cells but is inducible by activators that deliver a complete growth stimulus to the T cell. The combination of PMA and ionomycin is capable of mimicking those signals required for T cell

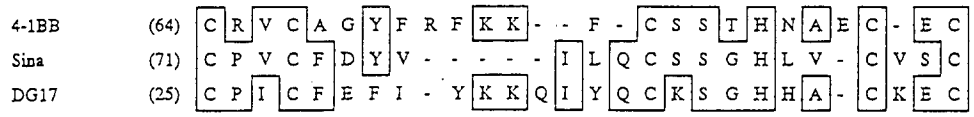

FIGURE 9. Comparison of the 4–1BBP amino acid sequence with the amino acid sequence in *sina* of *Dorsophila* and DG17 of *Dictyostelium*. The amino acids that are shared are *boxed*. *Numbers* represent the positions of the left-most residues relative to the N-terminus. Gaps (-) are introduced to allow for maximal alignment.

proliferation. Although PMA or ionomycin alone induced 4–1BB mRNA, the combination of PMA and ionomycin resulted in optimal 4–1BB expression. Furthermore, the expression of 4–1BB was not transient. When purified splenic T cells were stimulated with immobilized anti-CD3, 4–1BB mRNA was expressed, and this expression was maintained for up to 96 h poststimulation. Cell cycle analysis will be required to confirm that 4–1BB is expressed throughout cell cycle progression.

4–1BB is structurally related to members of the NGFR superfamily. Although these receptors possess structurally similar ligand-binding properties (cysteine-rich regions), the cytoplasmic domains of these proteins are nonconserved, which could allow for diversity in transmembrane signaling. Some members of this family are involved in the T or B cell activation process. There are in vitro functional data on the OX-40, CD40, and CD27 Ag. Antibodies against the OX-40 Ag augment the T cell response in a mixed lymphocyte reaction (7), whereas antibodies against CD40 both enhance B cell proliferation in the presence of a coactivator (such as PMA or CD20 antibodies) and synergize with IL-4 in vitro to induce B cell differentiation and to generate long term normal B cell lines (29). One mAb, anti-1A4, which recognizes an epitope on the CD27 molecule, inhibited calcium mobilization, IL-2 secretion, Th cell function, and T cell proliferation (11). On the other hand, CLB-CD27/1, another anti-CD27 mAb, enhanced proliferation of human T cells stimulated with PHA or anti-CD3 mAb (28). These results indicate that the CD27 molecule plays an important role in T cell activation. Except for TNFR, NGFR, and CD40, the ligands or cell surface molecules to which the members of the superfamily bind are not yet identified. We are presently using a 4–1BB-alkaline phosphatase fusion protein to search for potential cell surface ligands that bind to 4–1BB. The identification of cell surface and/or soluble 4–1BB ligands will be helpful in defining the physiologic role of 4–1BB.

To ascertain whether cell surface 4–1BB could contribute to T cell activation, we used the anti-4–1BB mAb, 53A2, as an agonist to 4–1BB. These data suggested that 4–1BB does in fact have the potential to function as an accessory signaling molecule during T cell activation and proliferation. The addition of soluble 53A2 mAb to purified splenic T cells stimulated with immobilized anti-CD3 resulted in an amplification of [^{3}H]thymidine incorporation compared to T cells stimulated with anti-CD3 alone. This pattern of enhancement ranged from 2- to 10-fold in three independent experiments.

In the original two-signal model of Bretscher and Cohn, it was proposed that signal 1, the occupancy of the TCR, resulted in inactivation of the T cell in the absence of signal 2, which is provided by accessory cells. This has since been confirmed by a variety of studies (30). The identification of CD28 as a potent costimulatory receptor on T cells was a significant contribution in beginning to characterize the accessory signal(s) required for optimal T cell proliferation (31). It is possible that other cell surface molecules may contribute to these costimulatory activation requirements (32).

We are interested in determining how 4–1BB contributes to T cell activation. The biochemical signals delivered through 4–1BB are unknown. One possibility is the observation that 4–1BB contains a putative p56^{lck} tyrosine kinase binding domain in its cytoplasmic tail. In fact, we have found that this tyrosine kinase binds to 4–1BB.[4] It will also be worthwhile to determine if 4–1BB-mediated signaling can regulate genes such as IL-2 and IL-2R, whose expression is required for T cell activation and subsequent proliferation.

Although the precise functions of members of the NGFR family appear to be diverse, an emerging theme is one in which these molecules may contribute in various ways to a maintenance of responsiveness or viability of the particular cell type in which they are expressed. For instance, nerve growth factor is absolutely required for the viability of neurons in vitro and in vivo (33). The cross-linking of CD40 by soluble anti-CD40 mAb blocks germinal center centrocytes from undergoing apoptosis in vitro (34). Signals delivered through CD40 may also induce a metabolic state of competence in which the B cell can now respond to differentiation factors. The ligation of CD40 with anti-CD40 F(ab')$_2$ fragments in the presence of IL-4 induced large increases in IgE synthesis (35). Also, anti-CD40-activated naive B cells treated with IL-10 and transforming growth factor-β became committed to IgA secretion (36). It will be exciting to investigate how 4–1BB may contribute to the maintenance of responsiveness at the activational as well as the differentiative level

[4] Kim, Y. J., Z. Zhon, K. E. Pollok, A. Shaw, J. B. Bolen, M. Fraser, and B. S. Kwon. 1993. A T cell antigen 4-IBB associates with the protein tyrosine kinase p56^{lck}. *Submitted for publication.*

in $CD4^+$ and $CD8^+$ T cells.

In addition to sharing the molecular characterics with the NGFR superfamily, we noted that the 4–1BB contained a putative zinc finger structure of the yeast eIF-2β protein (37). 4–1BB also shares a conserved region with the *sina seven in absentia* of *Drosophila*, which is required for correct photoreceptor cell development (38). That particular region is also similar to the protein product of the DG17 gene of *Dictyostelium*, whose expression is specifically induced during aggregation by cAMP (Fig. 9) (39). This region forms the pattern of $C\text{-}X_2\text{-}C\text{-}X_9\text{-}C\text{-}X_3\text{-}H\text{-}X_3\text{-}C\text{-}X\text{-}C$; and the cysteines and histidine are conserved in a similar space in 4–1BB, *sina*, and DG17 proteins. Ten of 24 amino acids between the 4–1BB and *sina* proteins are identical. Between 4–1BB and DG 17 proteins, 11 of 24 amino acids are identical, and 3 of 24 are conservative substitutions. The conserved pattern suggests that these amino acids are functionally important. The *sina* protein is localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4–1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4–1BB may play diverse roles during cellular proliferation and differentiation.

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